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SNAIL STOMACH CYTASE, A NEW REAGENT FOR PLANT CYTOLOGY

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ABSTRACT.—**A very active mixture of cytase and other enzymes is present in the stomach fluid of *Helix pomatia*, the edible snail. This fluid makes an ideal macerating medium for squash preparations of root-tips and other similar plant tissues. The complete separation of all cells is easily achieved, while the chromosomes remain completely unaffected. The method of using this fluid is described; it can be applied in conjunction with any of the standard fixatives and stains used in making squashes.**

Treatment with normal HCl is the most usual way of softening tissues such as root-tips for making squash preparations of chromosomes. This process may at the same time produce the hydrolysis of desoxyribose nucleic acid necessary for the Feulgen stain (Heitz, 1936). Alternatively, mixtures of HCl and alcohol (Warmke, 1935) or of HCl and acetic acid stains (Darlington and LaCour, 1942) have been used. Hillary (1940) on the other hand recommends 4% ammonia. In all these cases the principal effect is on the calcium pectate of the middle lamella, the cellulose walls being more resistant and generally remaining intact. A serious drawback of all these methods is that the reagents used are strong, and if allowed to act for too long, quickly affect the chromosomes. With most materials it is impractical to continue maceration for long enough to obtain the complete separation of all cells, and in such cases one may resort to splitting the root-tip, or cutting it into sections. Nevertheless, the margin of safety between sufficient maceration on the one hand and damage to the chromosomes on the other is not large. Many workers consider it impossible to make consistently good squash preparations of certain material, such as root-tips of grasses.

The fluid in the stomach of the Roman or edible snail, *Helix pomatia*, contains a concentrated and remarkably powerful mixture of enzymes. The properties of this fluid have been known for a long time (Yung, 1888). Biedermann (1911) in particular has

described these properties in detail, and a more modern account of the enzymes present in the secretion is given by Karrer, Staub, Weinhagen and Joos (1924). Many enzymes have been identified: diastase, invertase, inulase, maltase, cellobiase, lipase and, what is important for the present purpose, the mixture of enzymes collectively designated as cytase, which breaks down cellulose and related substances. Another remarkable and important property is the apparently total absence of any proteolytic activity. Owing to this last fact the stomach fluid can be used directly without preliminary purification.

The method of obtaining the stomach fluid is as follows: A snail is killed by immersion in chloroform for a few minutes. The shell is then removed and the mantle cavity opened. The "stomach" will at once be apparent as a translucent tube filled with a coffee-colored liquid. This fluid is easily extracted if one end of the digestive tract is pulled out and opened. One active *Helix pomatia* yields between 1 and 1½ ml. of fluid. Snails in the dormant state must first be made active by keeping them warm and feeding them for a few days. If a drop of toluene be added, the fluid will keep its properties for at least two months at room temperature; it is likely that preservation in a refrigerator is better still, but this has not been tried. According to Biedermann (1911) the fluid can also be dried without losing its effectiveness.

If this reagent were to be applied on the scale usual in cytological technic, at least one snail would be needed for a single preparation. Actually this is quite unnecessary. By using small glass vials 10 or 12 mm. high, and of an internal diameter of about 3 mm. (which are easily made out of thin walled glass tubing) it is possible to macerate about a dozen root-tips together, using no more than about 20 cu. mm. of fluid. Thus at least fifty preparations of a dozen root-tips each can be made with the stomach secretion of one snail. Such a microtechnic is no more difficult, and at least as rapid as ordinary methods. It is essential to prepare some strips of wood with holes in them for standing the vials upright, and a number of fine fountain pen fillers for emptying and filling them.

To obtain rapid action the author has preferred to use the fluid undiluted, but for some purposes dilution may be desirable. All material that has been tried was fully macerated overnight at room temperature. No harm, however, results from leaving it much longer. In one trial a batch of root-tips was left for two weeks, and at the end of that period yielded good preparations when stained in Feulgen. The pH is not critical, and there is no need to use a buffer.

Snail enzyme maceration has been successfully used after the following fixatives: 3 parts alcohol to 1 part glacial acetic acid, Navashin, medium Flemming, 2 BE, and 2% OsO_4. After fixatives containing OsO_4, it is essential to remove traces of osmium by treatment with dilute H_2O_2, as otherwise enzyme action is inhibited; ordinary washing seems sufficient after CrO_3 fixatives. Maceration is applied immediately after fixing and washing; previous storage in 70% alcohol does no harm, but the alcohol must be washed out first. Lanthanum acetate does not inhibit the enzyme.

Feulgen stain and the preliminary hydrolysis can be applied with equal success either before or after maceration, as the snail secretion does not dissolve the stain. Normally, however, hydrolysis and staining after maceration are preferable. Carmine, orcein and lacmoid in 45% acetic acid have also been used very successfully. The making of the squash preparation follows the usual methods. After maceration the root-tip is flabby but holds together if handled gently with tweezers. It is placed on a slide in a drop of 45% acetic acid, or acetic stain as the case may be, and covered with a cover slip which is afterwards tapped to break up the root-tip. The preparation is then pressed hard between layers of filter paper. Heating is usually desirable at this stage. If the cover slip has been previously smeared with albumen, the preparation can be made permanent in the usual way by opening in 45% acetic acid, passing through alcohol-acetic mixtures, and mounting in Euparal or other medium.

After this process most of the cell-walls disappear, but the chromosomes remain embedded in a sponge-like mass which probably consists of the cytoplasmic proteins. Such preparations are very well suited for flattening out the chromosomes by strong pressure in order to reveal details of structure; in some materials at least, this final flattening is best done at the Euparal stage.

Snail enzyme maceration has been successfully tried on the following: root-tips of *Secale, Zea, Trillium, Galtonia, Pancratium, Uvularia;* embryos of *Phaseolus* and *Brassica;* pollen mother cells of *Paeonia* and *Tradescantia.* There is no special advantage in using it on pollen mother cells, although the cell wall is dissolved. Snail secretion will not attack the wall of ripe pollen grains, and so cannot be used for pollen grain divisions. Owing to the diastase present, starch grains always disappear completely, which may at times be an advantage.

Snail stomach enzyme maceration is probably too troublesome for strictly routine purposes. The object of this paper is not to

describe a new technic, but to draw the attention of cytologists to a new reagent which would seem to have many possible uses other than those enumerated here. Its outstanding property is its effectively unlimited safety margin, enabling it to be used so as to effect the complete separation of all non-lignified cells. It seems probable that the chemical nature of fixed chromosomes is unaffected, and the extremely clear preparations obtainable may, for example, have their use in ultra violet absorption work.

The writer has not tried snail species other than *Helix pomatia*, but it seems very likely that some of them will do just as well (Biedermann, 1911). It may be worth pointing out that *Helix pomatia* can be kept for some months in the laboratory simply by being fed with cabbage or lettuce. It can also be made to breed in captivity, but in that case a supplementary diet is necessary (Howes and Whellock, 1937).

With a different purpose in view, cytase extracted from fungi has been used for clarifying chromosome preparations, by Stuart and Emsweller (1943).

I wish to express my gratitude to Mr. L. LaCour for much valuable assistance, and to Mr. N. W. Pirie for first suggesting that the snail might be a suitable source of cytase.

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ROMANOVSKY STAINING OF LOW VISCOSITY NITROCELLULOSE SECTIONS OF BONE MARROW

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In the detailed study of bone marrow, sections of undisturbed marrow are essential in determining the distribution and relative proportion of the various hemopoietic cells and the relative volume occupied by marrow cells, fat, and blood in the various portions of the same bone or of different bones. As a result of trial of a number of fixatives, stains, and embedding technics, the following technic was evolved. It gives the best combination of good differential staining with the least shrinkage and cellular distortion, and is used routinely by the author in the study of bone marrow lesions in rats.

One femur and a short segment of dorsal vertebrae are stripped of soft tissues, fixed 48 hours in Orth's fluid, washed in running water 18 hours and decalcified in 5% aqueous formic acid. A thin transverse block of spinal column and a longitudinally sectioned half of the femur are dehydrated in ethanol (80%, 8–16 hours; 95%, 8–16 hours; 2 changes of absolute, 8–16 hours), placed in 1:1 ethanol-ether for 2 hours, placed in 10% low viscosity nitrocellulose (in 1:1 ethanol-ether) for 1 week, placed in 30% low viscosity nitrocellulose (in 2:3 ethanol-ether) for 1 week, embedded in 30% low viscosity nitrocellulose in a closed Stender dish under a bell jar until firm, hardened in chloroform 2 hours, trimmed and mounted on fiber blocks, placed in 1:1 chloroform-cedar-oil for 24 hours, placed in 1:3 chloroform-cedar-oil for 24 hours, placed in pure cedar oil 24 hours, wiped dry, and sectioned dry at 8 μ in a sliding microtome. The sections are passed through 2 changes of 95% ethanol to remove cedar oil and are stored in 80% ethanol.

A modified Romanovsky stain is used. Sections are brought to water, stained 5 minutes in Delafield's hematoxylin diluted 1:2 with water, dipped in acid-alcohol (1% HCl in 95% ethanol), cleared in 95% ethanol, blued in 1% aqueous disodium phosphate, rinsed in 2 changes of distilled water and stained 1 hour in the following: 2 ml. of azure-eosinate (0.3% in 1:1 glycerol-methanol), 46 ml. distilled water, 1.1 ml. M/10 citric acid, 0.9 ml. M/5 disodium phosphate. Sections are then differentiated and dehydrated in 2% colophony in 95% ethanol. This step removes the stain from the nitrocellulose matrix. Following this, sections are floated onto

clean glass slides, blotted dry, flooded with xylene 3 times, blotted after each application of xylene, and mounted in 60% xylene-clarite.

The selection of azure-eosinate depends upon the staining effect desired. The author prefers B-eosinate of azure A or B-eosinate of toluidine blue. The latter was prepared by adding to 250 ml. 0.8% aqueous toluidine blue NU-3 (2 g.), 250 ml. 0.4% eosin-B NEb-9 (1 g.). The resulting precipitate of toluidine-blue-eosinate was recovered on a Buchner filter, washed 3 times with distilled water, air-dried, and dissolved in 1:1 glycerol-methanol, 300 mg. per 100 ml. Eosin B gives brilliant staining of eosinophiles. Azure A or toluidine blue gives excellent color balance in nuclei, cytoplasm, and mast cell granules.

OIL BLUE N OR NA AS A FAT STAIN FOR ANIMAL HISTOLOGY

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ABSTRACT.—**Oil blue N or NA gives precise dark blue staining of fatty substances in animal tissues when supersaturated solutions in dilute isopropanol are used. A stock saturated solution in 60% isopropanol, when diluted to 40 or 50% isopropanol, gives good staining in 5 to 10 minutes, without appreciable precipitate.**

Suitable counterstains are 1:1000 solutions of Janus green B, Bismarck brown Y and Bismarck brown R. These require about 5 minutes and should be followed by one-minute differentiation in 5% acetic acid. Of the three, Bismarck brown R gives the best contrast.

Recently the papers of Whittenberger (1944) and of Addicott (1944) on the use of oil blue NA (1,4-bisamylaminoanthraquinone) as a differential stain for rubber in plant tissues came to our attention. As, on reading these papers, it seemed probable that this dye might serve as a useful stain for fat in animal tissues, a sample was procured through the courtesy of the Calco Chemical Division of the American Cyanamid Company.

It was first tried in the supersaturated isopropanol technic of Lillie and Ashburn (1943), using frozen sections of normal cat kidneys, adrenals and other tissues, and gave a sharp blue stain to fats. However, there was an excessive amount of crystallin precipitate due to the relatively great difference in solubility in 99% and in 60% isopropanol. Apparently the solubility in 99% isopropanol is in the neighborhood of 2.5% since 450 mg. was filtered out of a several day old suspension of 3 g. in 100 ml.

Since it was observed that 2 ml. of this saturated solution in 250 ml. water gave a bluish metallic scum and a fairly deep blue solution, a few frozen sections were left overnight in this water, and light blue staining of fat was obtained. This gave rise to the hope that successful fat stains could be obtained from considerably lower concentrations of isopropanol than the 60% currently in use for oil red O (1944).

Dilutions of the stock 99% isopropanol saturated solution to 70, 60, 50, 40, 30, 20 and 10% isopropanol levels were made. With the first, considerable loss of fat was noted; with the last, unduly pro-

longed staining periods were required, while good stains were obtained in 10–20 minutes in the 60 to 20% levels.

As it would be convenient if the stock solution could be used directly rather than after dilution and filtration, saturated solutions in 60, 40, and 20% isopropanol were tried. The first gave satisfactory stains in 20–30 minutes, but it was further observed that longer exposures, up to 2 or 3 hours, occasioned notable loss of fats. The lower isopropanol levels required prolonged staining.

It was then found that when a stock saturated solution in 60% isopropanol was diluted to 40 or 50% isopropanol content by addition of water and the resultant supersaturated solutions used during the first few hours after dilution, intense blue staining of fats was obtained in 5 to 10 minutes, and little or no precipitate was seen.

A second dye sample, furnished through the courtesy of the National Aniline Division, performed in the same manner as that from the Calco Chemical Division. This second sample of 1,4 bisamylaminoanthraquinone was designated as oil blue N.

For a counterstain it was necessary to find a basic dye which would stain nuclei vigorously and selectively in contrasting color and would not be extracted by syrup mounting media. The safranins diffused badly into the mounting medium, the fuchsins even more rapidly; neutral red, chrysoidin Y, rhodamine B and pyronin B failed to stain nuclei selectively or bled badly. The samples tried were safranin O, C.I. No. 841 (NS-11 and NAC 10881*), methylene violet, C.I. No. 842 (3RA; Farbwerke Hoechst), safranin MN, C.I. No. 843 (6B, NAC 10071*), pararosanilin chloride, C.I. No. 676 (NAC 9155*), basic fuchsin, C.I. No. 677 (NF-31), new fuchsin, C.I. No. 678 (HL, about 1930), rhodamine B, C.I. No. 749 (NAC 10321*), pyronin B, C.I. No. 741 (Biosol 62), neutral red, C.I. No. 825 (HL, about 1930), and chrysoidin Y, C.I. No. 20 (NAC 9241). Three dyes appeared to be quite satisfactory in that they did not bleed appreciably into the modified Apathy gum syrup employed (See Lillie and Ashburn, 1943). These are Janus green B, C.I. No. 133 (HL, about 1930), Bismarck brown Y, C.I. No. 331 (NN-6), and Bismarck brown R, C.I. No. 332 (NAC 11044*).

After some trial and error, it was found best to stain 5 minutes in 1:1000 aqueous solution and then to differentiate 1 minute in 5% aqueous acetic acid solution. Janus green B gave sharp gray green nuclei and pale greenish cytoplasm, Bismarck brown Y a rather light but quite precise brown with pale brownish yellow cyto-

*Samples furnished through the courtesy of the National Aniline Division.

plasm, and Bismarck brown R a deeper and redder brown precise nuclear stain with similar cytoplasm.

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AN OXIDATION REDUCTION METHOD FOR STAIN DIFFERENTIATION[1]

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The difficulty in differentiating carmine-stained tapeworm proglottids is well known to those who have undertaken the task. Ordinary methods of differentiation, with 0.5% or 1% HCl, leave considerable amounts of color on the external tissues, a fact which not only is objectionable, but also renders it difficult to discern the morphology of internal organs clearly. We have found a method by which the external tissues are rendered colorless, yet the internal organs remain well stained.

The principle of the method employed is not new, but to our knowledge, its application for differentiating stained tissues is new. Heneguey[3] first described the method as a bleaching technic. The principle of the method employed utilizes the oxidizing capacity of $KMnO_4$, to destroy the carmine on the surface layers of the proglottid, and the products of oxidation are reduced by the action of oxalic acid. Its application for stain differentiation, with slight modification of the original formula, is enumerated below.

1. Boil saturated $HgCl_2$ solution and pour on live suspended tapeworm.
2. Fix in $HgCl_2$ solution for six hours.
3. Wash in tap water overnight.
4. Dehydrate to 70% alcohol and place in alcoholic iodine (5 g. iodine in 100 ml. 70% ethyl alcohol) until no further change of color occurs.
5. Rehydrate and stain for ten days in aqueous picro carmine.[4]
6. Dehydrate stained proglottids and differentiate in 1% HCl in 70% alcohol until proglottids appear translucent.
7. Determine number of proglottids to be placed on each slide and cut the tapeworm.

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[1]The author is indebted to Doctors J. H. Bodine, R. L. King, and L. O. Nolf.

[2]Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

[3]Lee, A. B. The Microtomist's Vade Mecum, *10th Ed.* Edited by Gatenby, J. B., and Cowdry, E. V. Blakiston, Philadelphia.

[4]Albert E. Galigher. 1934. The essentials of practical microtechnique in animal biology. Berkeley, California.

8. Pour 1% $KMnO_4$ in a watch glass and place a section into it directly from 70% alcohol.[5]

9. Examine the section at two-second intervals until it appears dark brown. It must not turn black.

10. Quickly dip in water and place in 10% oxalic acid solution for 5 to 10 minutes or until the proglottid appears white or faintly pink.

11. Rinse in tap water.

12. Dehydrate in alcohol.

13. Clear in clove oil.

14. Rinse in xylene or toluene.

15. Mount in 70% clarite.

The results obtained are excellent. Since the penetration of $KMnO_4$ and oxalic acid into the proglottid is slow, only the stain on the body surface is affected. The external structures are rendered "glass-clear" while internal structures remain a dark rose color. The technic is easily achieved, and the yield of well-differentiated proglottids is 100%.

This method was also used for large whole mount chick embryos, in which the extra-embryonic membranes were cleared and then double-stained with indulin. It should be interesting to try this method of differentiating stained tissues on the various Medusae and colonies of Hydrozoa.

[5]The $KMnO_4$ must be changed frequently.

A PHYSIOLOGICAL APPROACH TO PERFUSION-FIXATION OF TISSUES WITH FORMALIN

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ABSTRACT.—**A method of perfusion-fixation with formalin is here presented which is based upon well established physiological principles. It represents an attempt to preserve more accurately the structure and relationships of nervous tissue found in the living state. The essential points include the following: (1) Perfusion at an hydrostatic pressure equivalent to the mean arterial pressure of the animal to be fixed to maintain the normal patency of the vascular bed. For the guinea pig, it is approximately 70 mm. Hg; for the cat and monkey, it is approximately 120 mm. Hg. (2) The addition of a colloid to the perfusion fluid which will exert an osmotic pressure equal to the hydrostatic pressure in the capillaries and thus prevent edema. Gum acacia is used in this method. Its concentration depends upon the hydrostatic pressure to be used: for 70 mm. Hg, in the guinea pig it is 2.4%; for 122 mm. Hg, in the cat and the monkey, it is 5.6% gum acacia. (3) The addition of an electrolyte to the perfusion fluid to make it isotonic to the tissue fluid and thus prevent a disturbance in the distribution of water in the animal with resultant tissue distortion. NaCl (0.9%) is used in this method. (4) For further fixation or for preservation of the tissues, they are placed in a solution of 10% formalin which contains 0.9% NaCl to minimize swelling.**

INTRODUCTION

The preparation of animal tissues for microscopic study always presents a difficult problem to the histologist and pathologist. Tissues have to be treated in such a way that their structure can readily be visualized under the microscope. Yet it is important that they be as little altered as possible during treatment if one is to obtain a view of their basic structure comparable to that of the living state.

The double duty of preserving tissue and preparing it for the processes to follow rests upon the fixation. Fundamentally, fixation

[1]Aided by a grant from The National Foundation for Infantile Paralysis to Northwestern University.

involves the denaturation or the coagulation of the tissue proteins. This prevents the post-mortem changes of autolysis and bacterial decomposition. The former it effects by inactivating the tissue enzymes, both intracellular and extracellular; the latter it effects by destroying the saprophytic bacteria that eventually reduce all dead tissues to their elementary chemical components. The importance of fixing tissues as soon after death as possible is well recognized.

Perfusion-fixation utilizes the vascular tree of the intact animal to bring the fixing fluid quickly and directly to every tissue (with the exception of tissues which have no direct vascular supply). In this way distribution of the fixing fluid by the relatively slow process of diffusion is limited to the minute region between capillary and adjacent tissue; a small quantity of fluid serves to fix in a brief interval of time. The practice in this laboratory is to cannulate the ascending aorta through an incision made in the left ventricle. An exit is provided for the fluid returning to the right side of the heart after it has traversed the systemic circulation by making an incision in the right atrium. The cannula communicates with two reservoir bottles by means of rubber tubing and a glass Y-tube. One bottle contains fluid designed to wash out the blood; the other contains the fixing fluid.

Recently it was observed that animals fixed in this manner with 10% formalin gained a considerable amount of weight. The increase in weight varied with the hydrostatic pressure under which the perfusion was carried out, i.e., with the height of the reservoir bottles above the animal. In general a greater increase occurred at higher hydrostatic pressures. It was found in a series of six guinea pigs that at a perfusion pressure of 90 mm. Hg a net increase of 13.4 to 19.6% in body weight took place. In a series of four guinea pigs perfused at a pressure of 60 mm. Hg the net increase in body weight ranged from 0.9 to 6.2%. The brain, which we were particularly interested in fixing properly, partook in this general increase in weight. Here also, the increase was greater at higher perfusion pressures. The average weight of unfixed brains of guinea pigs of the size used for this work is 3.8 g. The brains of animals perfused at a hydrostatic pressure of about 90 mm. Hg averaged 5.1 g. This represents an increase of 34% in the weight of the brain. At lower perfusion pressures the increase was somewhat less. All the soft tissues exclusive of the blood partook in this general increase in weight.

It became apparent that perfusion-fixation, as it is commonly

practiced, results in an increase in the mass of the perfused animal. This increase in mass comes from the filtration of the perfusing fluid into the tissues. A consideration of the physico-chemical principles underlying the distribution and mobilization of water and salts in the organism readily reveals the mechanism for this edema and points out the histologic distortion that may result from such edema. Though it is not our intention to discuss in detail the principles involved, some pertinent points will be made.[2]

In the living animal the exchange of materials between the blood and the tissue fluids takes place chiefly by diffusion across the capillary endothelium. This is a purely physical phenomenon which depends upon the relative concentration of a diffusible substance on both sides of the endothelium, diffusion taking place from the region of the greater to that of the lesser concentration. There is the additional factor of blood pressure within the vascular tree. This pressure tends to filter the permeable elements of the blood, i.e., all of the constituents of the blood except the formed elements and the plasma proteins, from the blood into the tissue spaces. Furthermore, the colloid osmotic pressure of protein in the tissue fluids also tends to attract water into the tissue spaces. Were these forces unopposed, the tissue fluid would be swelled at the expense of the blood volume. In the normal living animal, edema or increase in the interstitial fluid is prevented by a number of opposing factors: hydrostatic pressure of the tissue fluid, lymphatic drainage of tissue fluid back into the general circulation and the colloid osmotic pressure exerted by the plasma proteins. The result of the action of all of these factors is the maintenance of a fairly constant blood volume.

A consideration of these principles makes clear the mechanism involved in the increase in the weight of animals during perfusion with 10% formalin. The forces opposing filtration of fluid from the capillaries into the tissues, i.e., lymphatic drainage and colloid osmotic pressure of the perfusing fluid, are entirely absent. The inevitable result is edema of the tissues. It was reasoned that by adding a substance to the perfusing fluid which will exert an osmotic pressure adequate to oppose the hydrostatic pressure in the capillaries and which will not pass through the capillary endothelium, edema can be prevented. Gum acacia (gum arabic) fulfills these requirements (Meek and Gasser, 1918); so it was decided to add this substance to the fixing fluid.

That the prevention of edema is desirable becomes evident upon consideration of the effect of the perfusion fluid entering the tissue

[2]See text books by Best and Taylor (1943) and by Goodman and Gilman (1941).

spaces. If an isotonic solution is used, e.g., 0.9% NaCl, there is an increase in the interstitial fluid with a corresponding separation of tissue elements, cellular and non-cellular. There is no redistribution of intracellular and extracellular water for there is no osmotic gradient. If a hypotonic solution is used, the addition of it to the interstitial fluid will render the latter hypotonic to the intracellular fluid. Water will then pass into the cells until isotonicity is established and hydration and swelling of the cells will result. Thus, in addition to the separation of the tissue elements consequent to an increase in the interstitial fluid, there is also a distortion of the cellular elements. If the perfusion fluid is hypertonic to the body fluids, then there will result a dehydration of the animal. Both the intracellular and the interstitial fluid compartments will be diminished in volume. The resultant tissue distortion is primarily that of shrinkage.

Ten per cent formalin, a solution of formaldehyde gas in water, acts as a hypotonic solution. The formaldehyde rapidly distributes itself so that its concentration in the intracellular and interstitial compartments is equal. The remaining water then serves to dilute the interstitial fluid. The net result is an increase in interstitial fluid and a swelling of the cells. The increase in interstitial fluid can be prevented by adding a nonpermeable, osmotically active substance to the perfusing fluid and we chose the colloidal substance, gum acacia. The swelling of cells can be prevented by adding a suitable electrolyte to the 10% formalin to make it isotonic and for this we chose NaCl. In conjunction with these adjustments it is necessary to perfuse the animal at a pressure equal to the arterial pressure of the living animal if the life-like appearance of the blood vessels is to be maintained. If the perfusion pressure be too great, blood vessels will be distended or even disrupted; if the pressure be too small, the vessels will be underfilled.

Procedure and Results

The method was worked out first for the guinea pig. A series of blood pressure determinations was made on four animals anesthetized with chloralosane. The common carotid artery was cannulated and connected with a mercury manometer. The blood pressure of each animal was noted continuously over a period of 20–30 minutes. The mean blood pressure was found to vary from 60 mm. to 75 mm. Hg. It was therefore decided to perfuse the guinea pigs at a pressure of 65–70 mm. Hg. The following perfusion fluids were used:

Solution A Sp. gr. at 23° C. = 1.010		Solution B Sp. gr. at 23° = 1.096	
NaCl	0.9 g.	NaCl	0.9 g.
Gum acacia	2.4 g.	Gum acacia	2.4 g.
Water	96.7 ml.	Formalin (commercial)	96.7 ml.

In preparing these and other acacia solutions the ingredients were put together in a bottle and allowed to stand until the acacia was dissolved. Acacia in the form of tears was used and was allowed to settle to the bottom of the bottle or was enclosed in a cheese-cloth bag and suspended in the fluid. The solutions were finally filtered through coarse filter paper or through several thicknesses of gauze. It was necessary to add a preservative to the NaCl-acacia-water solution. Formalin in a concentration of 0.25% (0.25 ml. per 100 ml. of solution) was found to be satisfactory.

It will be noted that we have used undiluted formalin in our perfusion-fixative solution. Since the unopposed action of formaldehyde is to shrink tissues, caution must be taken when perfusing with full-strength formalin not to let the concentration of formaldehyde in the tissues reach too high a level. Not over 25 ml. of such fixing fluid should be allowed to run into guinea pigs ranging from 350 g. to 500 g. in weight; roughly comparable amounts should be used to fix animals of other sizes. Diluted formalin may be used, in which case more fixative may be run into the animal. With NaCl-acacia solution in 10% formalin an unlimited amount of fixing fluid may be run through the animal without appreciable shrinkage.

Five animals were perfused with solutions A and B with the reservoir bottles at a vertical distance of 825 mm. above the animals. With the specific gravity of these solutions taken into consideration, this is equivalent to an average hydrostatic pressure for the two solutions of about 65 mm. Hg. The results appear in Table 1.

TABLE 1. RESULTS WITH RESERVOIR BOTTLES 825 MM. ABOVE ANIMALS

Animal	Wt. before Perfusion	Wt. after Perfusion	Change
No. 20	636.3 g.	632.3 g.	−0.6%
No. 21	380.0 g.	377.2 g.	−0.7%
No. 22	541.5 g.	535.8 g.	−1.1%
No. 23	411.6 g.	405.3 g.	−1.5%
No. 24	387.3 g.	384.1 g.	−0.8%

Six other animals were perfused with solutions A and B, with the reservoir bottles at a vertical distance of 900 mm. above the animals. The hydrostatic pressure at this height averages about 70 mm. Hg. The results are found in Table 2.

TABLE 2. RESULTS WITH RESERVOIR BOTTLES 900 MM. ABOVE THE ANIMALS.

Animal	Wt. before Perfusion	Wt. after Perfusion	Change
No. 39	885.0 g.	882.0 g.	−0.3%
No. 40	558.7 g.	557.8 g.	−0.02%
No. 41	806.0 g.	785.0 g.	−2.6%
No. 42	482.5 g.	481.0 g.	−0.3%
No. 43	1074.3 g.	1077.2 g.	+0.2%
No. 44	779.6 g.	781.0 g.	+0.2%

For cats and monkeys the following fixing solutions were found to be effective in maintaining relatively constant the weight of the animal during perfusion, with the average hydrostatic pressure for the two solutions at 122 mm. Hg (reservoir bottles were 1560 mm. above the animals):

Solution C Sp. gr. at 23° C. = 1.023		Solution D Sp. gr. at 23° C. = 1.109	
NaCl	0.9 g.	NaCl	0.9 g.
Gum acacia	5.6 g.	Gum acacia	5.6 g.
Water	93.4 ml.	Formalin (commercial)	93.4 ml.

The experiments described so far demonstrated that formalin-fixation can be carried out without swelling or shrinkage of the nervous tissues. It was found, however, that tissues underwent swelling and hydration when they were removed from the perfused animal and placed in 10% formalin. The addition of 0.9 g. of NaCl to 99.1 ml. of 10% formalin, to make it isotonic, minimized this hydration. Tissues fixed in our laboratories by the above described method of perfusion-fixation are now routinely placed into 10% formalin containing 0.9% NaCl to insure their complete fixation and to preserve them until it is desired to process them further.

For the microscopic study of the brain, fixed in this manner, the brain was dehydrated in two changes of dioxane,[3] passed through ether-alcohol, ether-alcohol-nitrocellulose, embedded in nitrocellulose, sectioned and stained with thionin in buffered solutions (Windle, Rhines and Rankin, 1943). To compare these brains with brains

[3]It was found that this agent caused no greater shrinkage of nervous tissue than the lengthier passage through ascending concentrations of ethyl alcohol.

The brain may be removed from the perfused animal and placed directly into dioxane. In this way a guinea pig brain is ready for imbedding in four hours. The histological results are relatively good. If more accurate cytological detail is desired it is recommended that the brain be placed in the isotonic 10% formalin solution for 12 to 24 hours, depending upon the size, before dehydration.

from animals fixed by perfusion with hypotonic and hypertonic fixing fluids without a colloid, one group of animals was perfused with a 10% formalin, and another group was perfused with a fluid consisting of 5 g. NaCl, 10 ml. of formalin and 85 ml. of water. The brains of these animals were further processed in exactly the same manner as the brains of those fixed with the saline-acacia-formalin mixture. Comparative appearance of neurons is shown in the photo-micrographs.

The histologic differences between these brain preparations are striking. As predicted from theoretical considerations, the neurons of the brains fixed in the hypotonic 10% formalin appear swollen and distended, (fig. 3 and 4) as compared with similar neurons of brains fixed with the isotonic fixing fluid containing acacia (fig. 1 and 2). The tissue elements also appear more separated. Capillaries and small blood vessels are strikingly absent. Apparently, these collapse and the endothelial nuclei are not ordinarily differentiated from nuclei of other types of cells, e.g., microglia.

The brains of those animals fixed with the hypertonic fixing fluid consisting of 5% NaCl and 10% formalin show evidence of even greater distortion. Grossly the brains lost considerable weight and were shrunken in all dimensions. They weighed 13.2 to 28.9% less than that of the average guinea pig. The ventricles were greatly distended. Microscopically the neurons are greatly shrunken and distorted (fig. 5 and 6). The cells appear crowded together. More blood vessels appear to be patent in these brains than in the preceeding group of brains.

The brains of those animals fixed by the method described in this paper appear superior histologically in every respect. We believe that the structure of the individual cells and their relationships to each other and to their non-cellular environment in the living tissue are preserved in these preparations. The presence of an intact vascular bed creates an exceedingly striking appearance. Even the smallest capillaries are visible and their lumina are patent. The richness of the blood supply of the brain is graphically evidenced in these sections. This is undoubtedly due to the retention of gum acacia in the lumina of these vessels which in turn retains fluid in the blood vessels and thus preserves their patency during the process of fixation.

Many more guinea pigs, cats and monkeys than those specifically reported in this paper have been fixed by this method in connection with other research. The results have been the same as those reported here.

PLATE 1

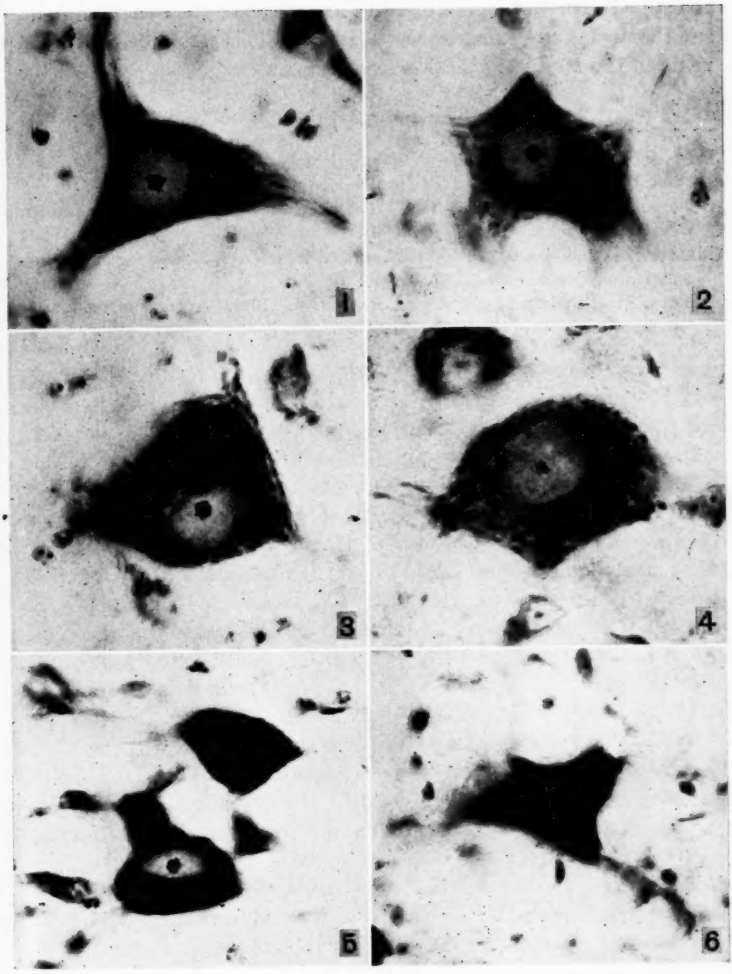
Explanation of Figures

Photomicrographs of large neurons of the reticular formation of the pons of adult guinea pigs. The sections were 40 μ thick and were stained by the buffered thionin technic at approximately pH 4.5. Magnification 390 $\times$.

Figures 1 and 2 show neurons fixed by the recommended perfusion-fixation method employing an isotonic formalin solution containing gum acacia.

Figures 3 and 4 show comparable cells from preparations by the old method of perfusion with 10% formalin, a hypotonic solution, resulting in general edema and in swelling of nerve cells.

Figures 5 and 6 show comparable cells from material fixed with a hypertonic formalin solution containing 5% NaCl. The nerve cells are severely shrunken.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The principles and method elaborated in this paper can no doubt be applied to perfusion fixation with fixing agents other than formalin and for tissues other than nervous tissues. This would be of especial value in the study of the blood vessels.

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WINDLE, W. F., RHINES, RUTH, and RANKIN, J. 1943. A Nissl method using buffered solutions of thionin. Stain Techn., **18**, 77–86.

A SIMPLIFIED NISSL STAIN WITH THIONIN

GEORGE CLARK and MARJORIE SPERRY, *Yerkes Laboratories of Primate Biology, Orange Park, Florida*

Recently two articles on the use of thionin as a cell stain for neurological materials have appeared. One utilizes a solution buffered in the acid range[3]; the other uses a "steaming" staining solution[4]. For some time we have been using thionin as a routine stain after either formalin or alcohol fixation and our method is so simple and has given such satisfactory results with a variety of brands of thionin that it seemed to be worthy of more general use. Briefly the method consists of placing the celloidin sections in a 0.05% solution of Li_2CO_3 (the percentage of Li_2CO_3 is non-critical) for about 5 minutes and then grossly overstaining in a 0.25% solution of thionin in a 0.05% solution of Li_2CO_3 in distilled water. The overstaining is necessary if all the stain is to be removed from the background. The sections are then passed through distilled water, 70 or 80% alcohol, two changes of butyl alcohol, two changes of xylene and mounted with Clarite. For most material, split mica cover-slips are quite satisfactory. The time of differentiation may be considerably lessened by the use of the differentiator recommended by Neumann (1942) except that we find the chloroform superfluous and transfer the sections to the aniline solution from 95% alcohol. Less fading seems to occur if the aniline differentiator is followed by a saturated solution of Li_2CO_3 in 95% alcohol.

With some brands of thionin it is unnecessary to add Li_2CO_3 to the staining solution. Thus we have at present five batches of dye on hand. Only two of these are certified (National Aniline & Chemical Co. certification numbers NT8 and NT9). One is an uncertified batch from Coleman and Bell, another is unknown and the last is presumably a Grübler product. It is necessary to add Li_2CO_3 to the staining solution when the two National Aniline and Chemical Co. products are used but not in the others. The Grübler dye stains the celloidin a green color that is difficult to remove. Satisfactory stains may be made from any of these dyes by this method, but the National Aniline and the Coleman and Bell products are slightly better and have a more intense violet color.

[3]Windle, W. F., Rhines, Ruth, and Rankin, James. 1943. A Nissl method using buffered solutions of thionin. Stain Techn., **18**, 61–8.

[4]Neumann, Meta A. 1942. A rapid method for the differentiation of nerve cells in old formalin fixed material. J. Neuropath. and Exp. Neurol., **1**, 348–50.

As a further check on the five batches of dye, comparative fading tests were made. Celloidin sections of rabbit medulla and cerebellum 50 μ thick were stained with each and then exposed for one and one-half hours at 27 inches to the ultraviolet rays produced by a Model F General Electric X-Ray Corporation machine. This would have produced a severe burn in the human. The Grübler product showed considerable fading and the background had turned the blue-green color typical of fading thionin. The unidentified product showed some fading, while the others were virtually unchanged.

NOTES ON TECHNIC

Plasma or Serum as a Diluting Fluid for Thin Smears of Bone Marrow

The usual smears of bone marrow are unsatisfactory because of the irregular distribution of cells and the occurrence of numerous thick clumps of cells which are so closely packed that they cannot be classified. Differential counts of such preparations are therefore of questionable significance.

These defects are easily overcome by smearing on slides a suspension of marrow cells in plasma or serum. Plasma or serum serves the dual function of isotonic diluent and protein matrix. (Physiological saline, Ringer's solution, and Tyrode's solution were found to cause distortion of cells and are not recommended.) Human plasma was found to be very satisfactory for diluting rat marrow, and is much easier to obtain than rat plasma or serum. A rubber stoppered vial of plasma or serum will keep well for several months if stored in the refrigerator. The only special equipment required is a supply of capillary pipettes. These are easily prepared by heating and drawing out glass tubing. A convenient size is prepared from glass tubing with an inside diameter of 5 mm. This is drawn out and cut in such a fashion as to form a capillary portion about 1 mm. in diameter and about 200 mm. in length, with a 200 mm. shank of unconstricted tubing.

Rats are killed by decapitation; the femur is quickly stripped of soft tissue and is cut open at the mid-shaft with heavy scissors. A capillary pipette is dipped into a container of human plasma. A column of plasma about 10 mm. long is allowed to rise in the pipette. The tip of the pipette is then introduced into the marrow of the femur and a 1–2 mm. column of marrow is aspirated into the pipette. The plasma and marrow are then blown out onto a clean glass slide and thoroughly mixed by repeated aspiration and expulsion. A small drop of plasma may be added if the suspension appears too thick. The suspension is then aspirated into the pipette and a small drop is placed on each of several clean glass slides. The drops are smeared by the usual blood film technic and the smears are allowed to dry. Excellent Wright, Giemsa, or peroxidase stains may be prepared from these smears.

The author prefers the following technic: Fix 3–5 minutes in absolute methanol. Allow to dry. Stain 30 minutes in a Coplin jar containing 1:50 aqueous dilution of Giemsa stain (800 mg. per

100 ml. glycerol-methanol) buffered to pH 6.0. Drain and allow to dry. Examine directly or mount in clarite using a large cover slip. Permanent well-stained uniform thin smears are thus obtained. A reaction of pH 6.0 is preferred to pH 6.5 because staining of hemoglobin is emphasized and depth of nuclear staining is decreased thereby facilitating differentiation between small lymphocytes and nucleated precursors of erythrocytes.—K. M. ENDICOTT, *Passed Asst. Surgeon, U.S.P.H.S., Pathological Laboratory, National Institute of Health, Bethesda, Md.*

A METHOD TO ELIMINATE OPACITY ON MOUNTING HOOKWORMS[1]

The majority of hookworms turn opaque as soon as they are immersed in balsam after being cleared. This peculiarity has been attributed to improper dehydration or to exposure to air between clearing and mounting. After some 700 individual experiments, over a period of three months, we wish to refute this misconception.

Hookworms were carefully dehydrated and cleared in xylene or toluene. To eliminate any possibility of coming in contact with air, the balsam was floated over the clearing oil, and the worms were gently drawn into the balsam. Ninety per cent of these worms turned opaque. It was noticed that those remaining clear were invariably ruptured. When an opaque worm was pierced with fine needles, bubbles of gas could be pressed out and the animal would clear.

In order to get all of the worms to stay clear after mounting, the hookworms were dehydrated to 70% alcohol, and their cuticles were pierced with fine needles under a Bausch and Lomb dissecting microscope. They were further dehydrated, cleared, and mounted without difficulty.

We believe that the balsam causes a very rapid exosmosis of the clearing oil so that a gas forms under the cuticle, causing opacity due to light diffraction.

This technic was employed on *Acylostoma caninun*, as well as *Necator americanus*.—THEODORE N. TAHMISIAN,[2] *Zoological Laboratories, State University of Iowa, Iowa City, Ia.*

[1]Aided by a grant from the Rockefeller Foundation for work in cellular physiology.

[2]The author is indebted to Dr. J. H. Bodine, who made it possible to carry on this work.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEW

LEGGETT, W. F. **Ancient and Medieval Dyes.** 5 x 8 in. 96 pp. Cloth. Chemical Publishing Co., Brooklyn, N. Y. 1944. $2.25.

This little booklet can hardly be regarded as a practical aid to the laboratory worker, but is nevertheless very interesting as showing the modern user of stains what dyes were available before the discovery of synthetic colors. A number of the products discussed are still in use, but many of them are almost unfamiliar names to the modern user of dyes. The author discusses the history of the following vegetable dyes: madder, indigo, woad, saffron, safflower, weld, brazilwood, logwood, camwood, the fustics, orseille, cudbear, annatto, turmeric, cutch, gambir and quercitron. Four animal dyes—Tyrian purple, kermes, cochineal, and lac—are mentioned, as well as one or two mineral dyes.—*H. J. Conn.*

MICROTECHNIC IN GENERAL

McCARTNEY, J. E. **A new immersion oil "polyric".** *J. Path. & Bact.*, **56,** 265–6. 1944.

A clear, neutral, non-drying, inexpensive, suitably viscous and optically correct immersion oil is obtained by selecting a mixture of the dimer and trimer of α-methylstyrene that furnishes the proper viscosity, and mixing at 60° C. with 2 parts of castor oil. This lowers the refractive index from 1.583 to 1.515. The polymethylstyrene is obtainable from B.X. Plastics, Ltd., Higham Station Avenue, South Chingford, London, E. 4.—*S. H. Hutner.*

NICKERSON, MARK. **A dry ice freezing unit for rotary microtomes.** *Science,* **100,** 177–8. 1944.

The construction and use of a unit for rotary microtomes is described whereby dry ice (solid CO_2) may be used in freezing either fresh or infiltrated tissues for sectioning. The method is recommended as useful in the preparation of a variety of tissues.—*T. M. McMillion.*

DYES AND THEIR BIOLOGICAL USES

BERGEIM, FRANK H., and BRAKER, WILLIAM. **Homosulfanilamides.** *J. Amer. Chem. Soc.*, **66,** 1459. 1944.

Several homosulfanilamides were prepared and tested for effectiveness in the treatment of experimental gas gangrene in mice. Of the compounds tested 4-homosulfanilamide and N′-methyl-4-homosulfanilamide showed the most protective action.—*A. P. Bradshaw.*

CALDWELL, W. T., TYSON, F. T., and LAUER, LOTHAR. **Substituted 2-sulfonamido-5-aminopyridines. II.** *J. Amer. Chem. Soc.*, **66,** 1479. 1944.

A series of new substituted 2-sulfonamido-5-aminopyridines and two new derivatives of sulfanilamide have been prepared. Pharmocological tests on these substances are now in progress.—*A. P. Bradshaw.*

JOHNS, C. K. **Dye concentration in resazurin tablets.** *Amer. J. Pub. Health,* **34,** 955–6. 1944.

One drawback to the widespread use of the resazurin test has been the necessity for weighing out accurately small quantities of the dye powder in the pre-

paration of a solution of the desired strength. Standardized resazurin tablets, produced by the National Aniline Division (Allied Chemical and Dye Co.) and approved by the Biological Stain Commission, are now on the market. These tablets contain a stronger concentration of dye than those manufactured in England. Although they show slightly more bacteriostatic effect, requiring about 30 minutes longer to reach the pink or white stages with the better grades of milk, the higher dye concentration does not appreciably affect the time required to reach the P 7/4 Munsell color standard used in the triple reading test. On the other hand, the stronger solution has an advantage in the greater ease of color differentiation, particularly with those milks which give a greyish tinge to the dye-milk mixture. Again, where the resazurin test is applied to cream, or to abnormal milks such as colostrum, there is a decided advantage in the use of a stronger solution.—*M. W. Jennison.*

SMITH, WINSLOW WHITNEY. **Relative sensitivity of different phases of growth curve of Bact. salmonicida to alkaline acriflavine.** *Proc. Soc. Exp. Biol. & Med.*, **56**, 240–2. 1944.

The organism showed variable resistance to 500 p.p.m. of alkaline acriflavine solution, more in the lag than logarithmic phases at 10° C. The solution is used on trout eggs.—*M. S. Marshall.*

VAN ARENDONK, A. M., and SHOULE, H. A. **Dialkylaminoalkyl derivatives of substituted quinolines and quinaldines.** *J. Amer. Chem. Soc.*, **66**, 1284. 1944.

A series of dialkylaminoalkyl derivatives of 4-amino-6-methoxyquinoline, 4-amino-6-hydroxyquinoline and 4-amino-6-methoxyquinaldine has been prepared. Pharmocological tests to determine if any of these compounds are suitably active in the treatment of malaria are to be reported upon elsewhere.—*A. P. Bradshaw*

WHEELER, KEITH, and DEGERING, E. F. **Preparation and properties of certain derivatives of sulfamide.** *J. Amer. Chem. Soc.*, **66**, 1242. 1944.

This paper presents the results of a dialkylsulfamyl chloride upon various amines. Over twenty derivatives of sulfamide have been prepared and their properties described. Some of these compounds, according to preliminary tests, have desirable physiological activity.—*A. P. Bradshaw.*

ANIMAL MICROTECHNIC

BOARDMAN, EDWARD T. **Methods for collecting ticks for study and delineation.** *J. Parasitology*, **30**, 57–9. 1944.

For greater ease in the study of small chitinized structures of ticks, the author made use of Galigher's modification of Bethe's method for testing for chitin (Galigher, A. T., 1934. The Essentials of Practical Microtechnique in Animal Biology, Berkeley, Calif., p. 241.) Materials were soaked 3–5 days in a solution containing 95 ml. of 90% alcohol and 5 ml. of concentrated HNO_3, washed in 3 changes of 50% alcohol, placed for 3 minutes in freshly prepared 10% aqueous solution of aniline hydrochloride, to which one drop of HCl had been added to each 10 ml. of solution, rinsed in water, and placed in 10% aqueous solution of $K_2Cr_2O_7$ until stained an intense grass green.

After rinsing in water the materials were transferred to water containing one drop of NH_4OH per 10 ml., whereupon the color soon changed to blue. After washing for 5 minutes in water, the materials were transferred to 70% alcohol. The chitinous parts of successful preparations were stained a clear semi-transparent blue. The length of time the materials were allowed to remain in the aniline hydrochloride solution and in the $K_2Cr_2O_7$ solution was varied according to the size of the structures to be stained.—*Elbert C. Cole.*

DICKIE, MARGARET M. **A new differential stain for mouse pituitary.** *Science*, **100**, 297–8. 1944.

A new technic is described for a reliable and consistent differential stain for the pituitary gland of the mouse, using a combination of several older methods. The material is embedded in a modified Zenker-formol (less $HgCl_2$ and formalin than normally), and is stained by a procedure based on MacFarlanes "picro-Mallory" technic (method I; see Stain Techn., **19**, 29–37. 1944).—*T. M. McMillion.*

GOVAN, A. D. TELFORD. **Fat staining by Sudan dyes suspended in watery media.** *J. Path. & Bact.*, **56**, 262–4. 1944.

Use of acetone or alcohol for solutions of fat dyes leads to loss of small fat globules and the dye is apt to crystallize out during differentiation. These difficulties are avoided by staining with the dye in aqueous dispersion.

The procedure used is as follows: Add a saturated solution of the dye (Sudan III, Sudan IV or Sudan black B) in acetone, drop by drop with a capillary pipette, into 1% aqueous gelatin containing 1% acetic acid. Stir vigorously throughout the addition to ensure rapid dispersal of the dye particles. (In general an equal volume of the acetone solution should be added to the gelatin solution.) Add the dye until the suspension of the precipitate which forms is deep brick red and resembles milk in consistency. Place in oven 2 hr. at 37° C. or in a warm room overnight to evaporate the acetone. (Temperatures above 37° C. tend to throw the dye out of suspension.) Filter through a coarse filter paper.

To stain, take frozen sections from water to 1% gelatin and leave 2–3 min. Stain with the filtered solution 30 min. at 37°. Wash in 1% gelatin 2–3 min. Wash thoroughly with water. Counterstain and mount as usual.—*S. H. Hutner.*

LILLIE, R. D., and ASHBURN, L. L. **Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degenerations not shown by Herxheimer technic.** *Arch. Path.*, **36**, 432. 1943.

A stock saturated solution of Sudan IV (NZ-11), oil red 4 B (Commonwealth Color and Chemical Co., No. 26870) or Sudan brown (Color index No. 81) is made in 99% isopropanol. For a day's staining, 3 ml. of stock solution is mixed with 3 or 2 ml. of distilled water for a 50 or 60% isopropanol solution respectively, allowed to stand 5 min. and filtered. For frozen sections: stain 10 min. in this solution (5 min. is sufficient in 60% isopropanol); float out in tap water; stain 2–5 min. in alum-hematoxylin containing 2% acetic acid; rinse in water; allow to turn blue in 1% Na_2HPO_4; mount in Apathy's gum syrup (50 g. of gum acacia and 50 g. sucrose dissolved in 100 ml. of distilled water at 55° to 60° C. with frequent shaking; 1.5 ml. of 1% merthiolate or 100 mg. of thymol added as a preservative; placed in a vacuum chamber for a few minutes to remove air bubbles). Fats are colored deep orange red by Sudan IV, a purer red by oil red 4 B, and a deep orange brown by Sudan brown.

Using the former staining technic, the authors observed a loss of fat from sections due to its solution in the high solvent concentration of the reagent. This difficulty is avoided by using 50% or 60% isopropanol as solvent; the tendency to precipitate formation in the staining mixture is also avoided.—*L. Farber.*

MULLEN, J. P. **A convenient and rapid method for staining glycogen in paraffin sections with Best's carmine stain.** *Amer. J. Clin. Path., Tech. Sect.*, **8**, 9–10. 1944.

The technic for demonstrating glycogen in tissue sections is as follows: Fix tissue blocks not more than 5 mm. thick 12–24 hours in absolute alcohol; cut blocks for sectioning and complete dehydration in two 4-hour changes of *n*-butyl alcohol; transfer to paraffin using 2 baths for about 12 hours and 1–2 hours respectively; embed as usual; cut sections 8–10 μ thick and float onto a glass slide; remove paraffin in xylene and place in absolute alcohol; cover sections with film of very thin celloidin (1:2 mixture of Mallory's "thin celloidin" and ether-absolute-alcohol); place in 80% alcohol to harden celloidin; stain with hematoxylin and Best's carmine, according to Mallory (Mallory, F. B., 1938, Pathological Technique. W. B. Saunders Co., Philadelphia. *See p. 127);* remove excess celloidin in a bath of 10% ether in absolute alcohol; clear in *n*-butyl alcohol and xylene; mount in balsam, Clarite or isobutyl methacrylate.—*L. Farber.*

NYKA, W. **A method for staining the rickettsiae of typhus in histological sections.** *J. Path. & Bact.*, **56**, 264. 1944.

The usual Giemsa stain for rickettsiae frequently yields irregular results. The following simple method gives uniformly good results: Fix small pieces of tissue in Müller's fluid. Embed in paraffin and cut thin (1–3 μ) sections. Stain 5–10 min. in 5% basic fuchsin in 90% alcohol. (This solution keeps well and sections may be left in it for days without affecting the differentiation.) Rinse rapidly in tap water; differentiate in 90% alcohol 1–2 min. Stain 1–3 min. in 1:10,000 methyl violet. Rinse in tap water. Differentiate thoroughly in 1:1500 acetic acid. Dehydrate in absolute alcohol or acetone. Clear in xylene and mount. Cytoplasm and red blood cells are pale yellow or pink; rickettsiae and nuclei, violet. Good sections were obtained from mouse lung and the yolk sac of chick embryos infected with typhus rickettsiae.—*S. H. Hutner.*

POPPER, HANS, GYORGY, PAUL, and GOLDBLATT, H. **Fluorescent material (ceroid) in experimental nutritional cirrhosis.** *Arch. Path.*, **37**, 161. 1944.

In this paper is included a method of staining liver sections with methyl green for demonstrating ceroid. The procedure is as follows: Stain paraffin and frozen sections of liver, fixed in 4% formaldehyde, 2 and 5 min. respectively, in 0.5% methyl green (National Aniline) in 20% alcohol; rinse in water or 1% acetic acid followed by water; mount in gum arabic (acacia). For mounting in Clarite the procedure is as follows: Stain overnight in a 1:10 dilution of 0.5% methyl green in 20% alcohol with 4% formaldehyde; rinse in water; treat 5 hours with 10% phosphotungstic acid solution; rinse in water; stain 3 min. in Van Gieson solution; dehydrate rapidly in absolute alcohol; clear in a 3:1 mixture of terpineol and xylene, followed by 2 changes of xylene; mount in Clarite.

A combined methyl green and fat-staining procedure for frozen sections of liver fixed in 4% formaldehyde is as follows: stain 5 min. in Herxheimer's solution (saturated solution of Sudan IV in 1:1 mixture of acetone and 70% alcohol) after dipping quickly in 70% alcohol; rinse quickly in 70% alcohol and in water; stain 2 min. in 0.5% methyl green solution in 70% alcohol; rinse in 1% acetic acid and in water; mount in gum arabic.—*L. Farber.*

SMALL, C. S., and SCHULTZ, M. A. **Restaining faded tissue sections.** *Amer. J. Clin. Path., Tech. Sect.*, **7**, 66–7. 1943.

Old and faded sections are restored to practically their original condition by the following procedure: Soak slides in xylene to remove cover slips (if section sticks to slide or cover slip, soaking in alcohol for a few minutes will loosen it). Place sections in 3 changes of 95% alcohol for 1–2 min. and blot gently; drop thin celloidin (1% in mixture of alcohol-ether, 1:1) over the sections and allow to run off at once. This treatment serves to keep them from falling off during subsequent manipulations. When celloidin is dry, place sections 1-2 min. in water; 1–2 min. in acid alcohol (1% HCl in 70% alcohol); 1 min. in water, with gentle agitation. Immerse in 0.1% ammonia water to remove old eosin, and rinse in water. Paraffin sections are then treated 1 min. with xylene; 1 min. with 98% alcohol; 1 min. with 70% alcohol; 1 min. with water; 4 min. with Harris hematoxylin; 1 min. in water; with 0.1% ammonia in 70% alcohol until hematoxylin becomes blue; 1 min. in 3 changes of 98% alcohol; 30 sec. in eosin alcohol (conc. not given); with carbol-xylene (no details given) until clear; 1 min. in 3 changes of xylene. Mount in gum damar. Frozen sections are stained 1.5 min. in the hematoxylin instead of 4 min.—*L. Farber.*

YOFFEY, J. M., and PARNELL, J. **The lymphocyte content of rabbit bone marrow.** *J. Anat.*, **78**, 109–12. 1944.

A technic is given for determining the cell content of marrow by placing a weighed quantity of marrow in a known volume of plasma and then breaking up the marrow. The cells become suspended in the plasma and the suspension is then treated much like blood to obtain total and differential counts. The marrow need not be weighed by itself. After an opening has been made in the bone

and an undetermined amount of marrow removed with a needle, and placed in the previously weighed plasma, the suspension itself is weighed and the marrow weight thus easily determined. The authors' studies were made on rabbit marrow, which contained 469,000 nucleated cells per cu. mm. of marrow (61,000 of them lymphocytes). Such a method, while relatively simple, will allow of quantitative studies on marrow by means of the smear rather than the section method. The specific gravity of red marrow has been found to be only very slightly more than 1.0, so that the weight in grams may be taken as equivalent to the volume in cubic centimeters.—*Warren Andrew.*

ZIEGLER, E. E. **Hematoxylin-eosin tissue stain. An improved, rapid, and uniform technic.** *Arch. Path.*, **37**, 68. 1944.

The recommended procedure is as follows: Dip sections, fixed in a 5–10% dilution of commercial formalin U.S.P., 8 times in xylene, second xylene, absolute ethanol, second absolute ethanol, 95% ethanol and isotonic NaCl solution; stain 6 min. in Harris' or Delafield's alum-hematoxylin solution; dip 8 times in isotonic NaCl solution, 4 times in 2% phosphotungstic acid in isotonic NaCl solution, 8 times in isotonic NaCl solution, 4 times in 2% sodium citrate in isotonic saline solution, and 8 times in isotonic saline solution; counterstain 2 min. in 5% eosin in 50% ethanol; dip 8 times in 95% ethanol, xylene, and second xylene; mount in balsam.

With this technic, requiring no more than 15 minutes, nuclei stain blue, cytoplasm pink to lilac, muscle tissue lavender, fibrous tissue pink, elastic lamina of blood vessels brilliant pink, and nerve tissue light grayish purple. A discussion of the reasons for the use of the various solutions is also given.—*L. Farber.*

PLANT MICROTECHNIC

HAASIS, FERDINAND W. **Staining rubber in ground or milled plant tissues.** *Ind. and Eng. Chem., Anal. Ed.*, **16**, 480. 1944.

In connection with work on the extraction of rubber from guayule, it was found helpful to examine the plants microscopically at various stages of the process to check its effectiveness. In working with ground material the following staining procedure may be followed: Place the sample in a small shell vial to cover the bottom to a depth of about 3 mm. To this add about 1 ml. of alkali solution (10 g. KOH + 100 ml. 95% ethyl alcohol). Mix well to be sure that all particles are wet. At the end of 15 minutes add approximately 2 ml. of bleaching solution (Clorox has been found satisfactory) and mix the preparation thoroughly. After standing 10 minutes wash the sample into a cone of tough filter paper and rinse with 3 funnelfuls of water. Return the sample to its vial and add about 0.5 ml. of Sudan IV solution (0.095 g. dye; 47.5 ml. acetone; 47.5 ml. 70% ethyl alcohol). After thoroughly mixing, allow the preparation to stand 30 min. Then wash the sample out into a Syracuse watch glass and drain off the water by capillary action of a small piece of bibulous paper. Wash the sample again in this manner, and while wet transfer to a microscope slide where it is collected into a small pile and excess moisture removed with another bit of paper. Add about 2 to 4 drops of mounting syrup (Karo, crystal white, is satisfactory) and mix thoroughly with the sample. After adding a cover glass, the slide is ready for examination.

Using this schedule, rubber is stained crimson, lignified tissues and cork cells bluish green or blue-green. If cutin is present, it stains a light pink or lavender color, not readily confused with the crimson of the rubber. Leaf veins are dull light green or blue-green.

This staining schedule may also be used in studying factory bagasse as a check on the completeness of rubber extraction in commercial milling.—*A. P. Bradshaw.*

PARRIS, G. K. **A simple nuclear stain and staining technique for Helminthosporia.** *Phytopathology*, **34**, 700. 1944.

The Helminthosporia are cultured on corn meal agar made according to Shear's formula. A clean Petri dish is lined both top and bottom with moist filter paper. A chemically clean glass slide is dipped in alcohol and burned in a flame, the melted corn meal agar is then poured over it and the excess drained

off until a thin even film covers the surface of the slide, which is placed on two glass rods in the Petri dish and, when cool, inoculated with Helminthosporia spores. As soon as the young conidiophores are present, they may be stained to show the nuclei by using Maneval's acid fuchsin stain: 5% aqueous phenol, 30 ml.; 20% aqueous glacial acetic acid, 8–10 ml.; 30% aqueous ferric chloride, 4 ml.; 1% aqueous acid fuchsin, 1–2 ml. The procedure is as follows: Add the above stain to the slide containing the fungal material. After 30 sec. to 3 min. drain off excess stain and wash one or more times in tap water, allowing water to stand on slide 3 to 10 sec. each time. When stain reaches the desired intensity, add two or more drops of lactophenol and cover with a cover slip. Examine under oil immersion lens. Nuclei are stained bright pink while the remainder of the cell is practically unstained. The agar does not retain the dye.

Lactophenol is prepared as follows: distilled water, 20 ml.; phenol crystals (melted), 20 ml.; lactic acid, 20 ml.; glycerin, 40 ml. Preparations mounted in lactophenol and ringed with paraffin will keep for months.—*Elizabeth F. Genung.*

MICROORGANISMS

DARZINS, E. **Rickettsienstudien.** *Zentbl. Bakt., Abt. I, Orig.*, **151,** 18–20. 1943.

The author describes a differential stain for Rickettsiae in chicken embryos. The mordant is prepared by diluting regular biological Lugol's solution to one half with distilled water. The staining solution is made as follows: dissolve 0.2 g. pure thionin in 10 ml. of 96% alcohol with constant stirring, bring volume to 100 ml. by adding distilled water gradually, filter. (This solution is at its best when 2 to 3 days old, but the mordant should be prepared just prior to use.) Spread tissue on slide uniformly and air dry. Apply mordant 30 sec.; rinse with distilled water; pour off water and, without drying, apply staining solution, 5 sec.; wash with distilled water, and dry.

The Rickettsiae are stained deep blue and are sharply outlined, the tissue near them is reddish-brown, the rest of the egg yolk appears to be brown and granular; young tissue is light green and Rickettsiae free; large developing epithelial cells are lemon yellow with blue to Burgundy red nuclei.—*C. Randall.*

GOHAR, M. A. **A staining method for Corynebacterium diphtheriae.** *J. Bact.*, **47,** 575. 1944.

Granules in **C. diphtheriae** may be stained as follows: Cover the fixed and dried film with Loeffler's methylene blue, 5 min.; wash with water; decolorize in a few seconds with 1:1000 H_2SO_4; wash with water; treat rapidly with Gram's iodine; wash with water; counterstain with 1% aqueous eosin, ½ min.; wash with water and dry. The organism appears pinkish, its granules blue-black.—*Virgene Kavanagh.*

GRAY, P. H. H. **Two-stain method for direct bacteria count.** *J. Milk Techn.*, **6,** 76. 1943.

A two-stain solution has recently been developed, with especial reference to differentiating capsules on bacteria. The solution has also been found to be applicable to staining milk films. The solution is prepared as follows:

A. 1% aqueous methylene blue	50	B. 1% aqueous basic fuchsin	25
Methyl hydrate	50	Methyl hydrate	25

Mix A and B.

Milk films, either of raw milk, or of pure cultures of bacteria in sterilized milk, are dried and treated (if not skim milk cultures) with xylene, which is washed off with methyl alcohol; the film is again dried and stained for a few seconds with the two-stain solution. Pre-treatment with xylene and methyl alcohol is not necessary with pure cultures in skim milk. A mixture of ethyl alcohol and tetrachlorethane in equal volumes may be used in place of the xylene and methyl alcohol. Bacteria and other cells are stained blue, serum solids and casein pink. The stain has an apparent advantage over the Breed and Newman-Lampert stain in that there is no eye-strain in picking out the cells; it may be considered to have an advantage over the Broadhurst-Paley stain in that it is easier to prepare.—*P. H. H. Gray.*